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PATHOGENICITY OF FOUR *FUSARIUM* SPECIES ON *ACACIA KOA* SEEDLINGS

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ABSTRACT

Fusarium isolates obtained from diseased koa plants, rhizosphere soil and seeds/seedcoats may or may not be pathogenic on young seedlings under greenhouse conditions. This includes isolates of *F. oxysporum*, the putative cause of koa wilt/dieback disease in Hawaii. We tested ten *Fusarium* isolates, comprising four different species, for their pathogenic potential on *Acacia koa* seedlings under greenhouse conditions. Tested isolates were obtained in Hawaii from either diseased *Acacia koa* seedlings, soil adjacent to seedling roots, or seeds/seedpods. All tested *Fusarium* isolates completely colonized seedling root systems and became systemic, spreading to above-ground plant tissues (stems, branches, and leaves). Virulence was quantified on the basis of production of disease symptoms (mortality, wilting, foliar chlorosis or necrosis) and effects on seedling height, diameter and root volume. Of the five tested *F. oxysporum* isolates, one exhibited high virulence, another was non-pathogenic, and the other three were moderately-virulent. One tested *F. solani* isolate was quite virulent, whereas the other was only slightly virulent. One isolate of *F. subglutinans* was non-pathogenic and the other tested isolate was moderately virulent on inoculated seedlings. The one tested isolate of *F. semitectum* displayed moderate virulence. Pathogenic screening of many more isolates, particularly those classified within the *F. oxysporum* species complex, will be necessary to identify pathogens that can be effectively used to screen families of *Acacia koa* for potential resistance to the wilt/dieback disease that is seriously impacting this important Hawaiian tree species.

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INTRODUCTION

Native *Acacia koa* A. Gray (Fabaceae) is a dominant canopy tree in many Hawaiian forest ecosystems. Koa is present on the main Hawaiian Islands, growing in moist habits at elevations from 90 to 2100 m. Koa has always been an important part of Hawaiian culture; koa wood was used for building many original Hawaiian structures, as well as being prized for sea-faring canoes. This important tree species is currently of primary importance in an expanding Hawaiian wood industry, being used for producing furniture, musical instruments, bowls, surf boards, and handi-crafts.

The major factor limiting establishment and maintenance of koa is a wilt/decline disease that adversely affects tree survival and growth. High tree mortality following koa plantings due to this disease has restricted koa establishment, particularly in certain areas where disease severity is high. Koa wilt/dieback is putatively caused by the fungus *Fusarium oxysporum* f.sp. *koae*, which was first described in Hawaii by Gardner (1980). Information on disease impacts and biology was subsequently investigated (Anderson and others 2002). Limited genetic analysis of several Hawaiian isolates of *F. oxysporum* indicated potentially low genetic diversity, possibly due to recent introductions of pathogenic strains of this fungus into the state (Anderson and others 2004). If pathogenic strains were recently introduced and non-native to Hawaii, the severe disease impacts currently occurring may be a reflection of an invasive, well-adapted, pathogen. Improved techniques for detection and management of this disease are urgently needed to limit pathogen spread and reduce disease impacts. Several other *Fusarium* species, particularly *F. solani*, are routinely isolated from diseased koa seedlings and trees (Daehler and Dudley 2002; James 2004). In addition, 13 different *Fusarium* spp. were recently isolated from koa seeds and seedpods (James and others 2006). Some of these organisms may be involved in disease etiology and epidemiology and require testing for their ability to induce disease.

Family variation in *Acacia koa* field trials strongly suggests presence of resistance to *F. oxysporum* f.sp. *koae* (Dudley 2002). Although wilt/dieback disease has significantly impacted koa plantings on certain sites, some seed sources appear mostly unaffected. Therefore, genetic disease resistance likely occurs and may be widespread within some families. Frequency of resistance in natural *Acacia koa* populations is unknown, but is expected to be low. Rapid screening of many koa families would provide valuable information on resistance potential that could be validated with outplanting performance tests. Inoculation methods for screening potential disease resistance in seedlings are available (James and others 1989; James 1996; Anderson and others 2002). Following laboratory and greenhouse experiments, field validation of resistance screening results will be needed (Sniezko 2004).

The present work was designed to evaluate pathogenic potential of selected *Fusarium* isolates associated with diseased koa plants and nearby soil in order to help clarify their potential roles in disease etiology. Tests were conducted under controlled conditions on koa seedlings within a greenhouse.

MATERIALS AND METHODS

Many isolates of *Fusarium* were obtained during routine isolations from koa seedlings, large trees exhibiting wilt/dieback disease symptoms, and seeds and seedpods. Isolates comprised several different *Fusarium* species (James 2004; James and others 2006), determined on the basis of morphological characterization (Nelson and others 1983). Some of these species have not previously been described on *Acacia koa* and their potential role in disease etiology is unknown. Therefore, seedling inoculation tests were initiated to help elucidate disease potential of selected representative isolates. Ten isolates encompassing four different *Fusarium* species were evaluated for their pathogenic potential (Table 1).

Fungal inoculum was prepared using the procedures of Miles and Wilcoxin (1984). Perlite, an inert, inorganic, siliceous rock of volcanic origin commonly used in potting mixtures, was the matrix for fungal growth. 150 g of yellow cornmeal was moistened with 300 ml warm 1% potato dextrose agar (PDA), to which 75 g of perlite were added. The perlite-cornmeal-PDA matrix was autoclaved at 121^{o}C for 60 min, cooled, inoculated with spore suspensions of test fungi, and incubated at about 24^{o}C in the dark for at least 15 days. After incubation, inoculum was dried in open petri plates within a cabinet. Inoculum dried within 5-7 days and did not become contaminated with other microorganisms because the food base was completely colonized by inoculated fungal isolates. Once dry, inoculum was refrigerated until needed.

Inoculum was ground to a fine powder and thoroughly mixed with commercial peat moss/perlite growing media (Sunshine Mix 4, Aggregate Plus, Sungro Horticulture, Belleview, WA) at a concentration of 1:50 (w/w). Inoculum-growing media mixtures were placed into plastic containers ("dibble tubes" – 115 mm^3) which had previously been sterilized by immersion in hot water (71^{o}C for 5 min).

Seeds of *Acacia koa* from one family were nicked at their distal end with nail clippers to break dormancy, soaked in water for about 12 hrs and sown into flats containing a 50:50 (v/v) mixture of vermiculite (Sta-Green Horticultural Vermiculite, St. Louis, MO) and perlite (Redco II, North Hollywood, CA), periodically watered, and monitored for germination. Following germination, when radicals were approximately the same length as cotyledons, they were carefully extracted from the flat and transplanted into the plastic containers containing inoculum-growing media mixtures. Following transplanting, seedlings were watered to activate inoculum. For each tested isolate, four replications of six seedlings each were evaluated. A fully replicated set of 24 seedlings was included as a control, which were transplanted into peat/perlite growing media without fungal inoculum.

Transplanted seedlings were monitored for development of wilt and/or foliar chlorosis and necrosis. When seedlings were considered dead, they were carefully extracted from plastic containers, their roots thoroughly washed to remove adhering particles of growing media, and analyzed in the laboratory for root colonization by inoculated isolates. For this analysis, ten root pieces, each approximately 5 mm in length, from each seedling, were surface sterilized in a 10% bleach solution (0.525% aqueous sodium hypochlorite; 1 part standard household bleach in 10 parts water), rinsed in sterile, distilled water, and placed on a selective agar medium for *Fusarium* spp. (Komada 1975). Plates were incubated under diurnal cycles of cool, fluorescent light at about 24^{o}C for 7-10 days. Emerging fungi were compared with inoculated isolates to determine whether they were the same morphological species.

At the end of the experiment (120 days), all surviving seedlings were examined for above-ground disease symptoms. Each seedling was rated for vigor based on severity of disease symptoms using the following numerical system: 0 = no disease symptoms; seedling appears healthy; 1 = seedling with less than 50% of its crown with wilt or foliar chlorosis/necrosis symptoms; 2 = seedling with greater than 50% of its crown with wilt or foliar chlorosis/necrosis symptoms, but still alive; 4 = seedling dead [entire crown with disease symptoms]. Seedling diameter just above the ground line and height from the ground line to the highest point of the main stem were measured on each surviving seedling. Seedling roots were washed thoroughly, surface sterilized as described previously, and analyzed for colonization by inoculated isolates as described above. Remaining roots were dried for at least 24 hrs at 100^{o}C and weighed (oven-dry weight = root volume). Means of seedling height, diameter, root volume and average vigor rating were compared and significant differences (P=0.05) found among isolates using the Waller Duncan-K Ratio t test (SAS Institute 1982).

A numerical system was developed to quantitatively compare virulence among tested isolates using all measured parameters: seedling

mortality, height, diameter, root volume, and vigor rating. This system assigned numbers for each parameter based on mean comparisons associated with each isolate, with lower numbers representing less fungal effects, i.e., less mortality, greater seedling height, diameter, root volume and higher vigor. Using this numerical system, *Fusarium* isolates were ranked on the basis of their overall virulence on *Acacia koa* seedlings.

RESULTS

Low levels of *Acacia koa* seedling mortality resulted from inoculating with most tested *Fusarium* isolates (Table 2). However, significant differences (P=0. 05) were found among different isolates for the other measured parameters, including seedling height, diameter, root volume and average vigor rating. When all parameters were collated, isolates were ranked as to their relative virulence on inoculated seedlings (Table 3). This ranking indicated that the five tested isolates of *F. oxysporum* varied widely in their relative virulence, including the most (0431B) and least (0429K) virulent of all evaluated isolates. The two isolates of *F. solani* we tested likewise exhibited relatively low (0424E) and high (0424I) virulence. One tested *F. subglutinans* isolate (0421A) was weakly virulent, whereas the other isolate of this species (0421J) was moderately virulent. The only tested *F. semitectum* isolate (0431J) was moderately virulent, although it was associated with the highest amount of seedling mortality (Table 2).

DISCUSSION

Fusarium isolates obtained from either diseased *Acacia koa* plants, soil adjacent to diseased plants, or seeds/seedpods varied widely in their virulence on inoculated koa seedlings under greenhouse conditions. This included several isolates of *F. oxysporum*, the putative cause of koa wilt/dieback disease. Apparently, not all isolates of this *Fusarium* species are equally virulent on koa seedlings, even though they may be isolated from diseased plant material. This is not surprising because isolates morphologically classified as *F. oxysporum* likely comprise several different biological species based on their genetic differences (Gordon and Martyn 1997; Kistler 1997; Di Peitro and others 2003). Large numbers of *F. oxysporum* associated with either healthy or diseased plants are likely saprophytic (Gordon and Martyn 1997; Bao and others 2002; Lori and others 2004); a much smaller number are capable of eliciting disease (Bao and Lazarovits 2001; Roncero and others 2003). Our results indicated that at least one tested isolate of *F. oxysporum* (0431B) was quite virulent on inoculated seedlings, whereas another isolate (0429K) was definitely saprophytic (Table 3). The former isolate was obtained from a disease koa seedling, whereas the latter was isolated from rhizosphere soil around the roots of a diseased plant. We plan to re-test both isolates in subsequent greenhouse inoculation trials to confirm their pathogenic or saprophytic behavior. The other tested *F. oxysporum* isolates were moderately virulent, indicating that these isolates were probably not aggressive pathogens, at least under our experimental conditions. They may be facultative pathogens that require high levels of plant stress to cause disease (Nelson and others 1981; Bao and others 2002).

We have routinely isolated high levels of *F. solani* from diseased *Acacia koa* plants (James 2004). This species is most often isolated from the interior of stems, branches, and large roots of plants displaying crown wilt/dieback symptoms, particularly from tissues adjacent to typical interior greenish-charcoal staining that is often associated with diseased plants. *Fusarium solani* has also commonly been associated with infestation by black twig borers (*Xylo-sandrus compactus* Eichhoff) [Coleo-ptera: Scolytidae], insects that routinely attack diseased trees (Daehler and Dudley 2002). The two isolates evaluated in this test displayed either relatively high or relatively low virulence (Table 3). Roles of *F. solani* in koa disease etiology are currently unknown, but may be important based on the relative frequency it is isolated from diseased plants. Further evaluation of its potential importance is warranted.

Fusarium subglutinans was commonly isolated from insect-predated seeds and seedpods collected from koa trees displaying wilt/dieback symptoms (James 2004). This fungal species has

previously been reported in Hawaii on plants in the genus *Aglaonema* (Uchida and Aragaki 1994). Isolates morphologically classified as this species may actually comprise several genetically-distinct species (Nirenberg and O'Donnell 1998;O'Donnell and others 1998). For example, two isolates obtained from diseased koa plants morphologically classified as *F. subglutinans* were genetically identified as *F. sterilihyphosum* Britz, Marasas & Wingfield sp.nov. (Hyphomycetes) (O'Donnell 2005), a species previously described only on malformed mangos in South Africa (Britz and others 2002). The two *F. subglutinans* isolates we tested were either non-pathogenic or exhibited either moderate virulence on koa seedlings. We suspect that this species (or species complex) may be associated with or vectored by seed-infesting insects and may not necessarily be important in disease etiology.

Fusarium semitectum is relatively common fungal species within subtropical or tropical environments (Nelson and others 1981, 1983). This species can be pathogenic on several different plant species (Onyike and Nelson 1992; Santou and others 2001; Dhingra and others 2002; Bokshi and others 2003) and often produces mycotoxins that may adversely affect plants and animals (Abbas and others 1995; Jimenez and others 1997; Logrieco and others 2002). The one isolate we tested was rated as moderately virulent on *Acacia koa* seedlings, although a quarter of the seedlings inoculated with this isolate died during our test. This relatively high mortality compared with other tested *Fusarium* isolates may have been because seedlings inoculated with this isolate were the last to be collected from germination trays. Therefore, they may have been the least vigorous of all tested seedlings. *Fusarium semitectum* is frequently isolated from disease koa plants and its potential role in disease etiology warrants further investigation.

We were able to identify at least one candidate *Fusarium* isolate (0431B) that may have some potential for screening *Acacia koa* families for resistance to the wilt/dieback disease. Our inoculation study indicated that *Fusarium* isolates obtained from diseased koa seedlings, adjacent soil, and seeds/seedpods are not necessarily pathogens. Five tested isolates of *F. oxysporum* showed a wide range of virulence on seedlings in our test. This would indicate that both pathogenic and nonpathogenic strains of this species, the putative cause of koa decline/wilt disease, commonly colonize koa plants. Therefore, all isolates of *F. oxysporum* obtained from diseased koa plants are not necessarily the pathogen capable of eliciting koa disease. Isolates will have to be genetically analyzed to locate potential markers associated with pathogenicity. Such markers may be effective to quickly and easily locate pathogenic strains from within fungal populations (Bao and others 2002; Lori and others 2004; Stewart and others 2004). Further pathogenicity tests, particularly with isolates of *F. oxysporum*, are planned to obtain additional isolates that can be effectively used for resistance screening. When mixtures of pathogenic strains are available, we should be successful in screening for disease resistance.

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Table 1. *Fusarium* isolates evaluated for pathogenicity on *Acacia koa* seedlings

Isolate Number	*Fusarium* Species	Isolation Location
0421A	*F. subglutinans* (Wollenw. & Reinking) Nelson, Toussoun & Marasas	Insect-predated koa seeds and seedpods
0421J	*F. subglutinans*	Insect-predated koa seeds and seedpods
0424E	*F. solani* (Mart.) Appel & Wollenw.	Stem of diseased koa seedling
0424I	*F. solani*	Stem of diseased koa seedling
0425K	*F. oxysporum* Schlechten.:Fr.	Roots of diseased koa seedling
0429K	*F. oxysporum*	Rhizosphere around roots of diseased seedling
0430B	*F. oxysporum*	Soil near roots of diseased koa seedling
0431B	*F. oxysporum*	Stem of diseased koa seedling
0433D	*F. oxysporum*	Roots of diseased koa seedling
0431J	*F. semitectum* Berk. & Rav.	Stem of diseased koa seedling

Table 2. Effects of selected *Fusarium* isolates on mortality, height, diameter, root volume, and vigor of inoculated *Acacia koa* seedlings.

Isolate Number	*Fusarium* Species	Percent Mortality[1]	Average Height[2]	Average Diameter[2]	Root Volume[3]	Vigor Rating[4]
0421A	*F. subglutinans*	0	30.6 BC[5]	38 B	0.63 AB	0.91 DC
0421J	*F. subglutinans*	0	22.9 E	30 E	0.53 BC	0.96 BCD
0424E	*F. solani*	0	29.2 BC	33 DE	0.52 BC	0.54 E
0424I	*F. solani*	0	23.8 E	30 E	0.48 CD	1.23 AB
0425K	*F. oxysporum*	4.2	24.3 DE	35 CD	0.55 BC	0.79 DE
0429K	*F. oxysporum*	4.2	35.6 A	42 A	0.73 A	0.59 E
0430B	*F. oxysporum*	0	27.2 CD	32 DE	0.52 BC	1.22 ABC
0431B	*F. oxysporum*	8.3	22.6 E	29 E	0.39 D	1.36 A
0433D	*F. oxysporum*	8.3	31.2 B	38 BC	0.61 AB	1.05 BCD
0431J	*F. semitectum*	25.0	28.3 BC	36 BC	0.52 BC	1.18 ABC
-	CONTROL	0	38.8 A	42 A	0.60 BC	0 F

[1]Percent of inoculated seedlings (24 per isolate) that died during the test (120 days)
[2]Average height (cm) and diameter (mm) at the end of the test.
[3]Average oven-dry weight (g) of root volumes at the end of the test.
[4]Average vigor rating at the end of the test; see text for descriptions.
[5]Within each column, means followed by the same capital letter are not significantly different (P=0.05) using the Waller-Duncan K-ratio t test

Table 3. Comparative virulence of selected *Fusarium* isolates on inoculated *Acacia koa* seedlings[1].

Isolate Number	*Fusarium* Species[2]	Mortality	Height	Diameter	Root Volume	Vigor	Virulence Rating
Control	-	1	1	1	2	1	6 - Nonpathogenic
0429K	*F. oxysporum*	2	1	1	1	2	7 - Nonpathogenic
0421A	*F. subglutinans*	1	2	2	1	3	9 - Low
0424E	*F. solani*	1	2	3	2	2	10 - Low
0433D	*F. oxysporum*	3	2	2	1	3	11 - Moderate
0425K	*F. oxysporum*	2	4	3	2	2	13 - Moderate
0430B	*F. oxysporum*	1	3	3	2	4	13 - Moderate
0431J	*F. semitectum*	4	2	2	2	4	14 - Moderate
0421J	*F. subglutinans*	1	5	5	2	3	16 - Moderate
0424I	*F. solani*	1	5	5	3	5	19 - High
0431B	*F. oxysporum*	3	5	5	4	6	23 - High
Maximum	-	4	5	5	4	6	24 - High

[1]Numbers in table compare averages for each isolate; higher numbers reflect greater virulence.